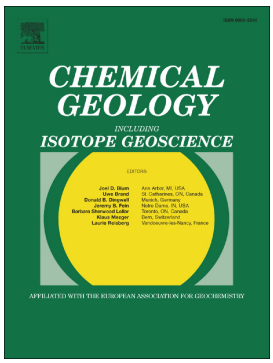
The Forgotten Variable: Impact of Cleaning on the Skeletal Composition of a Marine Invertebrate

Accepted Manuscript



The forgotten variable: Impact of cleaning on the skeletal composition of a marine invertebrate

Jennifer Loxton, Jens Najorka, Emma Humphreys-Williams, Piotr Kuklinski, Abigail M. Smith, Joanne S. Porter, Mary Spencer Jones

PII: S0009-2541(17)30586-7
DOI: doi:10.1016/j.chemgeo.2017.10.022
Reference: CHEMGE 18511

To appear in: *Chemical Geology*

Received date: 15 May 2017
Revised date: 12 October 2017
Accepted date: 17 October 2017

Please cite this article as: Jennifer Loxton, Jens Najorka, Emma Humphreys-Williams, Piotr Kuklinski, Abigail M. Smith, Joanne S. Porter, Mary Spencer Jones , The forgotten variable: Impact of cleaning on the skeletal composition of a marine invertebrate. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Chemge(2017), doi:10.1016/j.chemgeo.2017.10.022

The Forgotten Variable: Impact of Cleaning on the Skeletal Composition of a Marine Invertebrate

Jennifer Loxton[1*], Jens Najorka[3], Emma Humphreys-Williams[3], Piotr Kuklinski[3,4], Abigail M Smith[5], Joanne S Porter [2,3], Mary Spencer Jones[3]

[1] The Environmental Research Institute, University of the Highlands and Islands, Ormlie Rd, Thurso, KW14 7EE, UK.

[2] International Centre for Island Technology, Heriot-Watt University Orkney Campus, The Old Academy, Back Rd, Stromness, Orkney KW16 3AW, UK.

[3] Natural History Museum, Cromwell Rd, London, SW7 5BD, UK.

[4] Institute of Oceanology, Polish Academy of Sciences, PL-81-712 Sopot, Poland.

[5]Department of Marine Science, University of Otago, PO BOX 56, Dunedin, New Zealand 9054

ABSTRACT: For centuries, invertebrate collections have been subjected to various post-collection and curatorial cleaning techniques. Cleaning, however, may damage or even dissolve skeletal calcium carbonate and consequently influence any subsequent geochemical analysis. We investigated the combined effects of three cleaning variables: water (deionized and tap water), bleach (10% and 78%) and ultrasound (all for a range of durations), on the skeleton of *Flustra foliacea* (Linnaeus, 1758), a marine bryozoan. Treated and control carbonates were analysed both before and after cleaning, measuring: $MgCO_3$ in calcite (X-ray diffractometry and staining); organic:inorganic carbon ratio, using elemental analysis for total carbon by combustion and for organic carbon by acid dissolution and combustion. Treatment solutions were analysed using inductively coupled plasma atomic emission spectroscopy (ICP-AES) to detect any Ca^{2+} and Mg^{2+} that may have leached out. Significantly more weight loss and removal of $MgCO_3$ from calcite occurred in bleach concentrations of 10% or higher, especially in longer duration treatments and with use of

ultrasound. Specimens with higher initial $MgCO_3$ in calcite were especially susceptible to Mg leaching. We suggest that the interaction between bryozoan skeletal $MgCO_3$ and cleaning solutions is controlled by a combination of solution chemistry and reaction kinetics, and that when cleaning specimens prior to geochemical analysis, less is better.

Keywords: pretreatment; bleach; ultrasound; bryozoan; *Flustra foliacea*

1. Introduction

Since the mid-twentieth century, skeletal composition of marine taxa, such as the Foraminifera (Dowsett et al., 2011) and Mollusca (Cohen and Branch, 1992), have been used in paleoclimatology and paleoecology, as the skeletal carbonate of historic and fossil specimens has been assumed to record seawater chemistry and temperature from the time at which it was deposited (Lowenstam, 1954). In recent years, it has also been hypothesised that animals with variable $CaCO_3$ skeletal chemistry might act as a bellwether for climate change and ocean acidification (Fabry et al., 2009); it is likely that skeletal carbonate mineralogy may help to predict the degree to which species will be "winners and losers" in the future (Andersson et al., 2008). During an organism's lifetime Ca^{2+}, Mg^{2+}, and CO_3^- ions are extracted from seawater and skeletal carbonate is deposited, with $CaCO_3$ polymorph, cation ratios and stable isotopes reflecting both abiotic environmental conditions such as temperature (e.g. Chilanger, 1962) and biological factors such as development (Kuklinski and Taylor, 2009; Smith et al., 1998; Smith and Girvan, 2010), growth rates (Kuklinski and Taylor, 2009; Smith, 2007), and physiological fitness (Stanley and Hardie, 1998). It is this lifetime record which is of interest to scientists, but a variety of factors can confound it (Lombardi et al., 2010), including early sea-floor processes and diagenesis (Lombardi et al., 2010; Smith et al., 1992).

Even if a living organism has been freshly collected, its skeletal composition may be affected by: methods of fixation/preservation (Loxton, 2014), duration and conditions of storage (Steedman, 1976), cleaning, and pretreatment for removal of organic carbon (Smith et al., 2016). The effects of cleaning on invertebrate carbonate are poorly understood, but it is necessary to consider them. Millions of historic skeletal carbonate samples are archived in museum invertebrate collections; they are increasingly under scrutiny to provide information about very recent changes in sea-water and climate (e.g. Fabry et al., 2009). New specimens are daily added to collections; they may be used for future investigations. Most of these specimens have been rinsed in water, washed with detergents, scrubbed, bleached, ultrasonicated and/or dried as part of the curatorial process. To understand the potential importance of these specimens and their skeleton chemistry, it is necessary to examine the effects of cleaning processes on the skeletal mineralogy and chemical composition of invertebrates.

This study focuses on skeletal calcite from a marine bryozoan (*Flustra foliacea*). Bryozoans as a taxon are especially well characterised in terms of skeletal composition, and their mineralogy has been shown to correlate with sea-water conditions (e.g. Smith and Key, 2004; Stanley and Hardie, 1998; Lombardi et al., 2008). *F. foliacea* has variable $MgCO_3$ content in calcite (Borisenko and Gontar, 1991; Schopf and Allan, 1970; Taylor et al., 2009) and small spines on a delicate skeleton make it especially vulnerable to damage and dissolution. We investigate the common cleaning techniques of washing, ultrasonication, and bleaching in water on the $MgCO_3$ content in calcite, mass and inorganic/organic carbon in this delicate invertebrate. Is it good practice to wash a bryozoan? And if so, what's the best method?

2. Material and methods

2.1. Material, Collection, and Preparation

Flustra foliacea is a marine bryozoan, commonly found at 5-40 m water depth in areas of moderate flow around the British Isles. The mineralogical and chemical skeletal composition of *F. foliacea,* has been characterised in previous studies (Loxton, 2014; Taylor et al., 2009; Schopf and Allan, 1970; Borisenko and Gontar, 1991). The Ocean Biogeographic Information System (OBIS) reports that its distribution in the eastern Atlantic extends from 66°N to 45°N, from the White Sea (The Intergovernmental Oceanographic Commission (IOC) of UNESCO, 2010) to the Bay of Biscay (Hayward and Ryland, 1998). Each colony of *F. foliacea* consists of many hundreds of genetically-identical individual units called zooids,

filter-feeding lophophores regularly arranged in a shared calcium carbonate skeleton (zoarium). Zooids are arranged in bilaminar strap-like fronds; a colony can grow up to 20 cm tall and superficially resembles seaweed (Fig. 1A). *F. foliacea* was chosen here due to its large colony size, its morphology of naturally occurring genetic replicates (frond tips), and its delicate skeletal morphology, making it susceptible to damage and dissolution (Fig. 1B). It is the only readily available bryozoan species in the UK with these characteristics.

<<INSERT Fig. 1: single column image, colour on-line, B&W in print >>

Fig. 1: *Flustra foliacea*, a common marine bryozoan found in shallow waters of the temperate western Atlantic. A. Underwater photo of a living colony from offshore Scotland showing bilaminar frond morphology. Scale bar = approx. 10 mm. Photo taken by J.S.Porter. B. Scanning electron micrograph showing individual calcified zoaria and delicate spines. Scale bar = 100 µm.

A single large (25-30cm tall) colony of *Flustra foliacea* was collected by SCUBA divers on 27 October 2012 from a depth of 16 m at Saulmore Point, West Scotland (56.455021N, -5.413649W). The specimen was air dried after collection and processed within one month.

Specimens for treatment were taken from 90 branch tips – they are clones and can be assumed to have grown and lived in uniform conditions. To ensure uniform surface area, a 65 mm diameter punch was used to extract a disc from the centre of 78 dried branch tips. A single disc of this size contains about 200 individual zooids. Each of the 78 discs was weighed using a fine scale balance (accurate to 0.01mg) both before and after treatment. The rest of the branch tip in each case was retained for control measurements. Additional branch tips (N=12) were used for staining; tips were bisected with half of each tip undergoing treatment prior to staining, as specified in table 1, and the other half being stained as a control.

2.2 Experimental Treatments

Discs were subjected to treatments consisting of 26 combinations of cleaning techniques (water, bleach, ultrasound) at three durations (10, 60, 480 minutes), with three replicate discs in each treatment (Tab. 1 and Fig. 2). Experiments were carried out at 19.5°C in a thermally stable and well-cooled basement. Deionized water (pH = 6.53, undersaturated with respect to calcite) or tap water (Kensington, London; pH = 8.1, saturated with respect to calcite) were mixed with common household chlorine bleach ("Domestos" 40.5 g/L NaClO) at

concentrations of 0, 10 and 78%, which encompass the range normally used in cleaning bryozoan skeletal carbonate (e.g. Cheetham et al., 1969; Rucker and Carver, 1969;Sadberg 1971; 1973; 1975; 1977; 1983; Tavener-Smith and Williams, 1972; Poluzzi and Sartori, 1973; 1974; Bone and James, 1997; Machimaya et al., 2003; Taylor et al., 2009; Loxton et al., 2012; Kuklinski and Taylor, 2009; Smith & Clark, 2010; Schafer and Bader, 2008; Smith and Lawton, 2010; Bader and Schafer , 2005; Loxton, 2014; Loxton et al., 2014a; Loxton et al., 2014b) . Half the treatments were carried out in an ultrasonic bath (Headland Engineering Developments model M3.5, 220/240 volts, 2.5amps, single setting). Duration of treatment was 10 minutes and either 60 or 480 minutes (because the harsher treatments were found to completely destroy the material in the longer time period in some cases). pH was measured using a Voltcraft pH-100 ATC pH Meter which was calibrated using buffer solutions at pH 4 and 7 and is reported as accurate to $\pm$ 0.1pH for measurements between pH4 and pH10 and accurate to $\pm$ 0.2pH for measurements from pH 10-13 (Voltcraft, 2017). pH and temperature were recorded for the cleaning solutions at the beginning and end of each treatment.

<<INSERT Tab. 1: double column width>>

Tab. 1: Experimental design: cleaning treatments and geochemical analyses for branch tips of *Flustra foliacea.*

	TREATMENT				**ANALYSIS**			
	Solution	**Ultra-sound**	**Bleach (%)**	**Duration (min)**	**Mg (XRD)**	**Carbon (CHN)**	**Mg (stain)**	**Mg/Ca in solution (ICP-AES)**
1	Deionised water	No	0	10	x	x		x
2	Deionised water	No	0	480	x			
3	Deionised water	No	10	10	x	x		x
4	Deionised water	No	10	480	x	x	x	x
5	Deionised water	No	78	10	x			
6	Deionised water	No	78	60	x			
7	Tap water	No	0	10	x			
8	Tap water	No	0	480	x			
9	Tap water	No	10	10	x			
10	Tap water	No	10	480	x			
11	Tap water	No	78	10	x			

12	Tap water	No	78	60	x			
13	Deionised water	Yes	0	10	x			
14	Deionised water	Yes	0	480	x			
15	Deionised water	Yes	10	10	x	x		x
16	Deionised water	Yes	10	60	x	x	x	x
17*	Deionised water	Yes	10	480	*			
18	Deionised water	Yes	78	10	x			
19	Deionised water	Yes	78	60	x			
20	Tap water	Yes	0	10	x			
21	Tap water	Yes	0	480	x			
22	Tap water	Yes	10	10	x			
23	Tap water	Yes	10	60	x			
24*	Tap water	Yes	10	480	*			
25	Tap water	Yes	78	10	x			
26	Tap water	Yes	78	60	x			

* two samples were destroyed by the treatment so no post-treatment analysis was possible

<<INSERT Fig. 2: double column image, colour on-line, B&W in print >>

Fig. 2: Flow chart of experimental design: cleaning treatments and geochemical analyses for branch tips of *Flustra foliacea.*

2.3 Geochemical Analyses

Following cleaning treatment, discs were air-dried and weighed, then cut in half, as were the branch tips from which they had been removed. One half of each treated disc (treatment) and each corresponding branch tip remainder (control) were analysed for wt% $MgCO_3$ in calcite using an Enraf Nonius X-Ray Diffractometer (XRD) with an Inel 120° position-sensitive-detector and cobalt generated X-rays in the Imaging and Analysis Centre at the Natural History Museum, London. Bryozoan samples were powdered using a quartz pestle and mortar and affixed using a drop of acetone to single quartz crystal substrates (zero-background holder). In order to calculate wt% $MgCO_3$ in calcite, the position of the d_{104} peak was measured, assuming a linear interpolation between $CaCO_3$ and $MgCO_3$, accurate to within 2% on a well-calibrated instrument (Kuklinski and Taylor, 2009). A linear relationship

of d_{104} vs. composition exists between 0 and 17.4 wt% $MgCO_3$ in calcite (e.g. Mackenzie et al., 1983) and all analyses within this study fall within this range. Pure silica (Si) and silver behenate ($AgC_{22}H_{43}O_2$) on quartz substrate were used as the instrument calibration standard (Blanton et al., 2000, 1995).

For five treatments (0 and 10% bleach in deionized water, with and without ultrasound at two durations), the other halves were analysed for total inorganic and organic carbon using hydrochloric acid dissolution and elemental analysis (CHNS). Each sample was finely ground using a quartz pestle and mortar and divided into two subsamples of 5mg each. The first subsample was placed into a tin capsule and weighed. The second subsample was placed in a silver capsule, weighed and then repeatedly treated with dilute hydrochloric acid [0.5% - 8% v/v] until all inorganic carbon had been dissolved. Between treatments the powder was allowed to dry on a heated ceramic plate set at a low temperature to facilitate evaporation of the dilute acid. A Thermo Finnigan EA112 Elemental Analyser was used to combust all powders at temperatures exceeding 1200ºC in an oxygen-rich atmosphere to aid complete combustion; the resulting carbon oxides were separated chromatographically and quantified. The results for the first powder provided data on the total carbon content in the sample; the second subsample provided data on the organic carbon in the sample; subtraction of the organic carbon from the total carbon allows calculation of the inorganic carbon in the sample. Precision was measured on multiple analyses of aspartic acid throughout the run; N, C and H variability was measured as <1%. A secondary standard BBOT (2,5-(Bis)-5-tert-butyl-2-benzo-oxazol-2-yl)thiophene),was also analysed to quantify accuracy of the method, and gave a deviation of < l% from the expected values for carbon. Results are, therefore, considered accurate to within 1%.

The extra 12 branch tips were cut in half; one half was subjected to 10% bleach in deionised water with and without ultrasound (2 treatments x 3 replicates), the other remained as a control. After rinsing and drying, both control and treatment were etched in 5% acetic acid for 30 seconds, dried and then immersed in Titan Yellow dye (Choquette and Trusell, 1978) for 20 minutes. Titan yellow stain is specific for $MgCO_3$; it stains areas with > 3 wt% $MgCO_3$ in calcite a visible red. Staining was fixed using sodium hydroxide and specimens were imaged using a Zeisslight and visually compared.

The bleaching solutions used in five treatments (0 and 10% bleach in deionized water, with and without ultrasound at two durations) were retained, and analysed for Mg^{2+} and Ca^{2+}

content using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). 9.8 ml of the solution was acidified using 2ml of concentrated HNO_3 and 0.5ml of concentrated H_2O_2 to ensure any suspended material was dissolved prior to analysis. The solution was dried down on a heated ceramic plate prior to re-dissolution in 10ml of a 2% [v/v] HNO_3 solution. The resulting solutions were analysed on a Thermo iCap 6500 Duo ICP-AES. Spectra characteristic of the elements Mg and Ca were measured in order to quantify content of Mg^{2+} and Ca^{2+} in the solution (Piwoni-Piórewicz et al., 2017). An in-house standard was run alongside samples and found to be accurate to 5% for Ca^{2+} and 10% for Mg^{2+}. Results are, therefore, considered accurate to 10%.

2.4 Data processing

Wt% $MgCO_3$ in calcite and mass data were tested to see whether they approximate a normal distribution using Anderson-Darling normality tests. The Anderson-Darling test hypotheses are H0: data comes from a normal distribution and H1: the data does not come from the normal distribution. Data for wt% $MgCO_3$ in calcite was found to approximate a normal distribution allowing the null hypothesis to be rejected, whilst the data for mass cannot be used to reject the null hypothesis.. Homogeneity of variance for wt% $MgCO_3$ in calcite and mass were tested using Levine's test of equal variance; both passed Levine's test. As the criteria for parametric testing were satisfied, data were analysed using linear regression and generalised linear model (GLM) ANOVA with posthoc Tukey testing. The best-fit multiple regression model was determined through creation of the most complicated model (all factors and factor combinations) and stepwise deletion of non-significant factors and factor combinations until full significance was achieved for all remaining factors/factor combinations. Bootstrapping was achieved with the package "boot" (Ripley, 2013), resampling the full observation vectors for 1000 iterations. Relative importance of regressors in multiple regression was calculated using the "reclaimo" package (Groemping, 2013) in R (R Core Team, 2013) using the function calc.relimp. This function uses the LMG method to calculate R^2 partitioned by averaging over orders (Lindeman et al., 1980).

3. Results

3.1. Mass and Loss of Mass

Discs weighed between 3.51 and 7.05 mg prior to treatment (mean = 5.09 mg, Stdev = 0.87, N = 78), and 0 to 5.85 mg after treatment (mean = 3.04 mg, Stdev = 1.58, N = 78). All but one sample display weight loss ranging between 0.54 and 6.11 mg during the course of treatment (mean = 2.08 mg, Stdev = 1.590, N = 77, outlier excluded), which was from 11.64 to 100 % of initial weight (mean = 40.44, stdev = 29.05, N = 77, outlier excluded).

<< Fig. 3, single column, colour on-line >>

Fig. 3: Initial weight and final weight of samples after treatment, with symbols indicating treatment regime.

There is a strong statistically significant difference between mass loss observed following cleaning treatments (N = 6) using ultrasound versus those not using ultrasound (2-way ANOVA, ultrasound*treatment, F = 5.339, P = 0.0004). However, *post hoc* analysis shows this to only be significant for the 78% bleach x 60 mins treatment (Tukey, P < 0.0001). There is a strong statistically significant difference between mass loss observed following cleaning treatments (N = 4) using different concentrations of bleach (0%, 10%, 78%), (2-way ANOVA, bleach concentration*treatment, F = 25.19, P < 0.0001). Tukey *post hoc* analysis shows this to be significant between treatments conducted with 0% and 10% bleach (P < 0.0001) and with 0% and 78% bleach (P < 0.0001). There is no significant difference between treatments conducted with 10% and 78% (P = 0.426). There is a strong statistically significant difference between mass loss observed from cleaning treatments (N = 6) conducted for different lengths of time (10mins, 60mins, 480mins) (2-way ANOVA, time*treatment, F = 67.347, P < 0.0001) (Fig.3). Linear analysis for the effect of time for individual treatments shows this to be significant for treatments using 10% bleach without ultrasound (P < 0.0001, R^2 = 84.7%, $y = 0.72163 + 0.00384x$) and with ultrasound (P < 0.0001, R^2 = 87.05%, $y = 1.058 + 0.0224x$); and treatments using 78% bleach both without ultrasound (P < 0.0001, R^2 = 92.73%, $y = 0.6807 + 0.0443x$) and with ultrasound (P < 0.0001, R^2 = 93.39%, $y = 0.5467 + 0.0702x$). There is no significant relationship with time for treatments conducted with 0% bleach.

Multiple regression modeling indicates that five factors or factor combinations are significant in explaining 85.44% of mass lost during treatment: 1) bleach concentration*time*initial wt% $MgCO_3$ in calcite *ultrasound (LMG method, P < 0.0001, explains 77.86% of variance); 2) bleach concentration*initial wt% $MgCO_3$ in calcite (LMG method, P = 0.0040, explained 6.72% of variance); 3) bleach concentration (LMG method, P = 0.0033, explained 6.64% of

variance; 4) bleach concentration*ultrasound (LMG method, P = 0.0003, explained 6.51% of variance and 5) initial wt% $MgCO_3$ in calcite *ultrasound (LMG method, P = < 0.0001, explained 2.28% of variance).

3.2 Water Source and Cleaning Solutions

There was no statistical difference for mass loss (Fig. 4) or wt% $MgCO_3$ in calcite (Fig. 5) following cleaning treatments (N = 12) relating to the use of deionized versus tap water. In subsequent analyses, the results for deionized and tap water treatments were thus combined prior to statistical analysis for other factors.

<< Fig. 4, single column, B&W on-line >>

Fig. 4: Initial weight and final weight of samples after treatment, with symbols indicating water source: filled symbols indicate deionized water, hollow symbols indicate tap water.

<< Fig. 4, single column, B&W on-line >>

Fig. 5: Initial wt% Mg in calcite and wt% Mg in calcite lost during treatment, with symbols indicating water source: filled symbols indicate deionized water, hollow symbols indicate tap water.

Cleaning solutions changed during the course of the experiment, both in terms of pH and temperature. Samples with bleach added had high pH values (around 12) initially, which either stayed the same or decreased over the course of the experiment. The greatest pH change occurred in treatments with 10% bleach over 480 minutes, no ultrasonication, where the initial pH above 12 reduced to almost 10. In contrast, the water only treatments (0% bleach) showed an increase in pH over the course of the treatment, with ultrasonication enhancing the effect (Fig. 6).

Experiments were carried out at 19.5°C in a thermally stable and well-cooled basement. Temperature nevertheless increased in all ultrasound treatments (Fig. 6), especially those of longest duration. The greatest temperature increase (almost 30°C) occurred in treatments 14 and 20 (10% bleach, 480 minutes). This effect could be considered a confounding variable, but it is impossible to ultrasonicate samples without increasing their temperature, so we have treated the temperature change as a necessary part of ultrasonication.

<< Fig. 6, double column, black and white>>

Fig. 6: Changes to cleaning solutions during the course of treatment. Top graph shows temperature, middle graph shows pH and bottom graph shows Ca^{2+} in solution (left axis) and Mg^{2+} in solution. Temperature data is missing for the two treatments using ultrasound, deionized water and 78% bleach over 10 and 60 minutes due to scientist error. Hollow symbols show initial measurement and filled symbols show final measurement after treatment has concluded. Where only one symbol is visible it is because the treatment caused no change and the initial and final measurements are the same. Blanks for the bottom graph were conducted the same way as the bryozoan cleaning treatments but with no sample present.

Cleaning solutions analysed (5 treatments and 3 replicates) showed more Ca^{2+} and Mg^{2+} in solution after the experiment was finished in all cases except one (deionized water for 10 minutes showed a small, 0.34mg, decrease in Ca^{2+} after treatment). Initial Ca^{2+} and Mg^{2+} varied in the treatment solutions prior to treatment and this may be because the bleach contained some Mg^{2+} and Ca^{2+}. There is a statistically significant relationship between the treatment and the final amount of Mg^{2+} and Ca^{2+} in solution (MANOVA, $F = 7.206$, $P < 0.0001$) (Fig. 6), and a statistically significant correlation between final Ca^{2+} in solution and the mass lost from the sample in 2/5 of the treatments (deionized water with 10% bleach, 10 mins, $P = 0.0198$; deionized water with 10% bleach, ultrasonicated for 60 mins, $P = 0.0066$). Similarly, there is a statistically significant correlation between the final Mg^{2+} in solution and the mass lost from the sample in 4/5 of the treatments (deionized water, 10 mins, $P = 0.0424$; deionized water with 10% bleach, ultrasonicated for 10 mins, $P = 0.0089$; deionized water with 10% bleach, 10 mins, $P = 0.019$; deionized water with 10% bleach, ultrasonicated for 60 mins, $P = 0.0062$).

There is no statistically significant correlation between the Ca^{2+} in solution and the Ca^{2+} lost from the samples in any of the treatments but there is a statistically significant correlation between Mg^{2+} in solution and the Mg^{2+} lost from the sample in 3/5 of the treatments (Fig. 6). deionized water, 10 mins, $P = 0.0094$; deionized water with 10% bleach, 480 mins, $P = 0.0109$; deionized water with 10% bleach, ultrasonicated for 60 mins, $P = 0.0003$).

3.3 Carbon Content and Organic:Inorganic Ratio

In the untreated (control) samples, total carbon ranged from 20.57 to 23.85 mg (mean = 21.93 mg, Stdev = 0.66, N = 78) with the organic carbon concentration ranging from 39.53 to 90.03% of the total carbon (mean = 69.03%, Stdev = 10.8687, N = 69). There is significantly less organic carbon after treatment than before for all treated specimens (Paired T-test, $P < 0.0001$, T-value = 7.99) and the amount of organic carbon lost is statistically different for all treatments ($F = 6.95$, $P = 0.006$). There is no statistically significant difference in the amount of inorganic carbon before and after treatment.

3.4 $MgCO_3$ Content in Calcite

The range of wt% $MgCO_3$ in calcite found in untreated samples (from 7.7 to 13.5, mean = 9.6 wt% $MgCO_3$ in calcite, Stdev = 1.32, N = 78) falls within previously published mineralogical ranges for *F. foliacea* (Taylor et al., 2009; Schopf & Allan, 1970; Borisenko & Gontar, 1991). Many treated samples lost $MgCO_3$ from calcite over the course of treatment (Fig. 7).

There was no statistically significant difference in wt% $MgCO_3$ in calcite following cleaning treatment relating to the individual factors of water type, bleach concentration or treatment duration. There is a statistically significant linear relationship between the $MgCO_3$ lost during cleaning and the initial concentration of wt% $MgCO_3$ in calcite in the sample ($P < 0.0001$, $R^2 = 68.14\%$) (Fig. 7).

Multiple regression indicated that 77.45% of wt% $MgCO_3$ in calcite loss during treatment is explained by 1) Initial wt% $MgCO_3$ in calcite of samples ($P < 0.0001$) and 2) the combined effect of bleach concentration*time ($P < 0.0001$). The relative importance of these factors is 83.25% and 16.75% respectively.

<<INSERT Fig 7: initial Mg versus Mg-calcite lost>>

Fig. 7: Variation in Mg content in skeletal calcite (wt% $MgCO_3$) of *Flustra foliacea* before and after treatment, with symbols that show treatment regimes. Negative loss indicates that specimens gained $MgCO_3$ during the course of the treatment.

Staining of untreated specimens (Fig. 8A) showed that high-Mg calcite was predominantly located in the spines of *F. foliacea* with the zooid walls covered by an organic layer. After 1 hour of 10% bleach and ultrasonication most spines were lost and much organic material had been removed exposing the high-Mg calcite zooid walls (Fig. 8B). After 8 hours of 10%

bleach (no ultrasound), all spines were lost, and the high-Mg calcite walls were exposed and showing damage and thinning (Fig. 8C).

<< INSERT Fig 8: plate, full colour if possible>>

Fig. 8: Titan Yellow staining of specimens of *F. foliacea* highlighting the spatial distribution of $MgCO_3$ in calcite (red). A: untreated sample. High Mg-calcite spines are shown to be stained red, examples indicated with arrows; B: Specimen cleaned for 1 hour in 10% bleach with ultrasound. Spines are no longer present; C: specimen cleaned for 480 minutes in 10% bleach. Spines are no longer present.

4. Discussion

4.1 History of Specimen Cleaning

Curatorial cleaning procedures may be as simple as washing in fresh water prior to preservation, as has been recommended for marine invertebrates since the early 19th century (Graves, 1817), although it is also common, both historically and temporally, for more aggressive methods to be used. Cleaning procedures for vertebrates differ greatly from those used for invertebrates with methods for bone often including heat treatments (e.g. Roger & Daniels, 2002; Kamba et al., 2013), enzyme maceration (e.g. Simonsen et al., 2011), acids (e.g. Toombs & Rixon, 1959; Rutzky et al., 1994) and even beetles (e.g. Hall & Russell, 1933; Hefti et al., 1980) In contrast early guides for naturalists, such as the Manual for the Practical Naturalist (Anonymous, 1831), suggested the use of Pearlash, Potash and Lye, alkalines historically used for bleaching textiles, for the cleaning of invertebrate skeletons. Norman, a prominent bryozoan taxonomist, advocated the use of "Eau de Javille", also referred to as "eau de Javel" or "eau de Javelle" in literature, a weak (~5%) solution of sodium hypochlorite, now more commonly known as "Bleach". Norman (1903) observed that "Eau de Javille" is an aggressive substance to organic material that destroys not only soft tissues but also dissolves chitin, an often-beneficial result for taxonomists. The high-quality drawings and descriptions in earlier taxonomic works by Linnaeus, Hincks and others suggest that it is likely Norman's predecessors from the 1800s also employed cleaning, bleaching and preparation methods, however, as observed by Banta et al. (1973), these are rarely documented and rather passed down by word-of-mouth. These authors advocate the use of 5% bleach, "Eau de Javille", overnight to prepare specimens (Banta et al., 1973) and

bleaching to remove organic material continues to be used in many invertebrate studies up to the present day (e.g. Smith & Girvan, 2010; Smith & Clarke, 2010).

Ultrasonic cleaning was developed in the mid 20th century and works through the process of cavitation, the formation of bubbles or cavities in a liquid. It is the collapse of these bubbles which generates shock waves which impinge on the surface of submerged items and effectively scour them (Chedd, 1970). Ultrasonication for the cleaning of specimens has been used since at the 1960s, initially for fossil preparation (e.g. Stevens et al., 1960; Adams, 1968) and subsequently on Recent invertebrates (e.g. Dyrynda and Ryland, 1982), and is still recommended for the cleaning of invertebrates by the Natural Sciences Collection Association (Walker et al., 1999).

Since the late 20th century, some scientists have begun to suspect that bleaching and ultrasonication might have negative impacts on invertebrate calcium carbonate skeletons. Sandberg (1971), observed that "overzealous ultrasonic treatment may result in disintegrated specimens" and Taylor & Weedon (2000) described a a thin exterior layer of calcium carbonate granular fabric, which "in many specimens is wholly or partly lost during bleaching or cleaning". These scientists both give examples of impacts on the physical integrity of bryozoan skeletons, however do not specify whether this will result in impacts on mineralogy. Smith et al. (1998), however, assert that neither ultrasonication nor bleaching affects mineralogy results.

4.2 General Effects of Cleaning on Mass

All samples except one lost at least some mass after treatment. Some samples were dissolved entirely (both deionized water and tap water treatments using 10% bleach and ultrasonication for 480 minutes). Greater mass loss occurred with increasing bleach concentration, use of ultrasound, longer treatment time, and higher initial wt% $MgCO_3$ in calcite. These factors were found to be statistically significant both individually and in combination. The type of water used for dilution and rinsing was found to have no overall effect on sample mass loss, but deionized water with no bleach added caused considerably more mass loss than tap water, especially when combined with ultrasonication. This may be due to the difference in pH between tapwater (pH 8.1) and deionized water (pH 6.53).

Mass loss in these cleaning treatments may be explained by a combination of dissolution and disarticulation/abrasion. The change in solution pH and the presence of Ca^{2+} and Mg^{2+} in the

post-treatment solutions is evidence that dissolution occurred, whereas the increasing mass loss related to ultrasonication may point to abrasion or loss of delicate features such as spines.

Less calcium was found in treatment solutions than was stoichiometrically calculated to have been lost from the calcium carbonate of the sample. Calcium could be released from both the organic and inorganic components of a sample; Ca^{2+} is a vital component of cells, found within organelles for purposes including cell communication and triggering muscle contraction (Alberts et al., 2010). However, Ca^{2+} is only found in fairly low concentrations in marine invertebrate tissues (e.g. 5.24 mg ions/kg of lobster muscle; Robertson, 1961) . The concentration of Ca^{2+} ions released from cell breakage would therefore be expected to be a minor contributor to calcium in the treatment solution with the main source of calcium in treatment solution from $CaCO_3$. It is possible that part of the $CaCO_3$ may have been lost from the fragile treated specimens during rinsing, drying and/or handling which could explain why there was less calcium in the solution than expected.

The magnesium detected in the treatment solution, on the other hand, was higher than the Mg^{2+} lost from $MgCO_3$ during treatment; there was, however, a strong correlation between the mass lost from the sample and the magnesium detected in the treatment solution. Lombardi et al., (2011) suggested that organic tissues enveloping the bryozoan skeleton play an important protective role, preventing dissolution of the calcium carbonate skeleton. This effect has also been observed in other calcifying taxa (Ries et al., 2009) and the data from this study seems to concur, with $MgCO_3$ dissolution increasing as more mass, the majority of which was organic carbon, was lost.

More magnesium was detected in treatment solutions than was lost from $MgCO_3$, suggesting an additional source of Mg^{2+} ions in the solution. Mg^{2+} is present in high concentrations in cells (Alberts et al., 2010), bound primarily to G-actin, the protein responsible for cell movement (Barden and Remedios, 1985), and found in high quantities in bryozoan larvae (Santagata, 2007). As a result, high concentrations of Mg^{2+} would be released from organic material during cell lysis and protein disruption, increasing the Mg^{2+} in solution beyond that released from $MgCO_3$ alone.

4.3 Effects of Water Used in Rinsing and Dilution on Composition

To our knowledge, there has been no formal consideration of the effects of tap water vs. deionized water in rinsing and preparing of bleach solutions in the context of carbonate

geochemistry. Our results (Fig. 4, Fig. 5 and section 3.2) show that, despite the lower pH and undersaturation of deionized water compared to tap water, there was no overall significant effect of water used on wt% $MgCO_3$ in calcite, loss of mass, or ions in the solutions after treatment (Fig. 6). The addition of bleach to most of the treatments immediately increased the pH to above 10 (Fig. 6), so the only treatments where we might have expected a dissolution effect from deionized water would have been in the 0% bleach treatments. And indeed the pH of these treatments increased from 6.5 to about 7 in the non-ultrasound treatment and almost 8 in the ultrasonic bath (Fig. 6). This means that the acidic water was titrated by dissolution of the specimens, and in these few treatments, water choice did make a significant different to weight loss. Nevertheless, addition of any bleach to a treatment solution, as often occurs, overrides any initial pH difference caused by the water, explaining why water choice had no overall impact on mass loss or any other variable.

4.4 Effects of Bleaching on Composition

The effects of bleaching with sodium hypochlorite have been formally investigated. Tasch and Schaffer (1961), in their study on scolecodonts, observed that *Chlorox* (household bleach containing sodium hypochlorite) caused translucency of specimens and the dissolution of fragile components in some specimens. Somewhat later, Gaffey & Bronnimann (1993) investigated the effects of *Chlorox* on echinoids and green algae and found that a 5% solution caused no dissolution of mineral components of skeletons detectable with SEM, even after treatments of up to two weeks. More recently, Keatings et al. (2006) examined the effects of bleaching on ostracod valve chemistry and found no change in Mg/Ca following bleaching.

Effects of chemical oxidation, usually conducted using H_2O_2, on $MgCO_3$ have been somewhat more investigated. Both Marr et al. (2013) and Feldmeijer et al. (2013) found no difference in Mg/Ca for Foraminifera treated with short periods of chemical oxidation. Barker et al. (2003) however, saw reductions of up to 25% in Mg/Ca resulting from chemical cleaning, although specimens subjected to less than 20 minutes of oxidative treatment were observed to display an elevated Mg/Ca. Watanabe et al (2001) also found Mg/Ca to be reduced in coral aragonite with all pre-treatments. Conversely, Mitsuguchi et al. (2001) report an increase Mg/Ca in coral aragonite following oxidation. Krause-Nehring et al. (2011) found a mixture of losses and gains of Mg/Ca following pre-treatment of the bivalve *Arctica islandica*. Smith et al. (2016) go so far as to advise not bleaching or removing organic material to avoid geochemical alteration. Overall, in the literature, there is little consensus as

to the effect of bleaching or oxidation on $MgCO_3$ in calcite and the topic continues to incite debate.

Bleach acts through the dissociation of sodium hypochlorite (NaOCl) with water to form a strong oxidizing agent (HOCl) and raised pH. At higher concentrations more oxidizing agent is produced, resulting in a greater statistical chance of collisions with organic material and a faster rate of reaction (Moore, 2012) .

The high alkalinity works to disrupt cell walls (Alberts et al., 2010) while the oxidizing agent breaks carbon bonds in organic material to denature proteins (Klein, 2012). Together these processes remove organic material from a sample, the loss of which is the main contributor to total mass loss during treatment in this experiment (section 3.3). Some inorganic material was also lost during bleach treatments (section 3.3), an observation also reported by Taylor & Weedon (2000) in bryozoans, and Tasch & Shaffer (1961) in scolecodonts. Bryozoan calcium carbonate is deposited in a complex comprising protein threads around a template of organic cuticle and periostracum (Hall et al., 2002; Tavener-Smith and Williams, 1972) . In the Mollusca (Zuschin et al., 2003) and Crustacea (Inoue et al., 2008) this intraskeletal protein matrix has been shown to hold the calcium carbonate skeleton together; loss of this organic matrix during treatment could result in calcium carbonate loss (Banta et al., 1973) .

Our results (Fig. 3; section 3.1) showed that higher concentrations of bleach resulted in more mass lost from samples during treatment. Bleach removes organic carbon, so the ratio of organic:inorganic carbon decreased when bleach was added to the treatment solution and as the duration of bleach treatment increased. Previous studies (e.g. Smith et al., 2016; Barker et al., 2003) have noted that low concentrations of bleach, for short times, are useful and mostly harmless for both cleaning and pre-treatment to remove organic material, but we agree with those who suggest that the lowest concentration of bleach possible should be used and for short durations.

4.5 Effects of Ultrasound on Composition

Chedd (1970) explained that the effects of ultrasonic techniques on a material are poorly understood and remain at the "empirical suck-it-and-see level" (Chedd, 1970), although some studies have since been conducted into the effects of ultrasound on calcareous specimens, such as corals (Watanabe et al., 2001) and Foraminifera (Hodgkinson, 1991). In 30-minute experiments, Clark (1973) demonstrated that optimum cleaning of nano-fossils occurred very

rapidly and continued ultrasound after this point caused only damage to calcareous structures. Hodgkinson (1991) summarised the method as "damaging and largely uncontrolled cleaning" (Hodgkinson, 1991) after observations that any weakness in foraminifera tests is almost immediately fractured using ultrasonication and observed particular erosion of calcite crusts. Watanabe et al. (2001) assessed the effects of a range of pre-treatments, all of which included ultrasonic baths, on Mg/Ca in coral aragonite and found that in all cases Mg/Ca was reduced after treatment. In contrast, Stevens et al., (1960) advocated the use of ultrasonication for limited amounts of time (< 15 mins) for the cleaning of fossils, including fossilised bryozoans.

The production of shock waves through cavitation has been shown to disrupt and lyse cells and has an abrasive action on a sample surface (Chedd, 1970); this action could explain the increased mass loss we found, much of which was inorganic carbon, when ultrasonication is used in treatments (Fig. 3; section 3.1). The friction of the sound waves moving through solution (Chedd 1970) caused warming of the treatment solution in all cases, increasing temperature up to 48.5ºC, 28ºC above room temperature. Warming is an intrinsic co-factor with ultrasonication, and generally increases reaction rates as particles have greater energy and the number of collisions is increased. Additionally more colliding particles have sufficient activation energy for a reaction to occur (Moore, 2012), which can increase the rate of protein denaturation and cell lysis (Alberts et al., 2010). Sjoberg & Rickard (1984) found that increased temperature raised the dissolution rate of calcite through alteration of the reaction kinetics. The process of cavitation causes constant small explosive movements in the treatment solution (Chedd, 1970), increasing the rate of movement of solution over the substrate surface. Sjoberg & Rickard (1984) also found increased movement to increase the rate of calcite dissolution. The impact of temperature and solute movement on calcium carbonate dissolution kinetics may explain the inorganic carbon loss seen in this experiment.

4.6 *Influence of Initial $MgCO_3$ Content*

It was observed during the experiment that the higher the initial wt% $MgCO_3$ in calcite in the sample, the more mass was lost during treatment. Titan Yellow staining showed that the fragile spines of *Flustra foliacea* are made from high Mg-calcite. The higher wt% $MgCO_3$ in calcite in some samples therefore may indicate that they have more of these spines intact. Spines are one of the first skeletal features to be lost during treatment, so samples with more spines to lose therefore exhibit greater mass loss than those with fewer spines to start with.

This effect may also partially explain the correlation between mass-loss and Mg^{2+} in solution. An alternative explanation could be the different solubility product constants (K_{sp}) for $CaCO_3^{calcite}$ (K_{sp} = 3.36 x 10^{-9}) and $MgCO_3$ (K_{sp} = 6.82 x 10^{-6}), which determine that $MgCO_3$ is more susceptible to dissolution than calcite (Arvidson et al., 2003). This, however, would only explain the small proportion of mass loss which is from dissolution of inorganic calcium carbonate.

Samples with higher initial wt% $MgCO_3$ in calcite generally lost $MgCO_3$, whereas samples below 8.73 initial wt% $MgCO_3$ in calcite were more likely to gain Mg during treatment (Fig. 7). This relationship was found to be the same regardless of whether tap or deionized water was used (Fig. 5). This is best explained by solution equilibrium chemistry. The chemical equilibrium for movement of Mg^{2+} between calcite and solution is shown in equation 2.

Equation 2: movement of Mg^{2+} between calcite and solution, x = mole fraction of Mg in solid phase, (Oomori et al., 1987)

$$Ca_{(1-x)}Mg_xCO_{3\,(solid)} \leftrightarrow (1-x)Ca^{2+}_{(soln)} + xMg^{2+}_{(soln)} + CO^{2-}_{3\,(soln)}$$

If the concentration of Mg in calcite on the left of the equation is higher than the concentration of Mg^{2+} ions in solution, then the reaction will be driven to the right, causing a reduction in wt% $MgCO_3$ in calcite. Conversely if there are more Mg^{2+} ions in solution than in calcite then the reaction will be driven to the left, increasing the wt% $MgCO_3$ in calcite (Oomori et al., 1987).

4.7 Effect of Treatment Duration

Sample mass loss was shown to be statistically increased with increasing treatment duration, probably also related to reaction kinetics – during a longer treatment duration more collisions occur between particles, and although the reaction rate would be unaffected (Moore, 2012), increased time would result in more total bond breaks in organic material and greater subsequent organic mass loss. The relationship between treatment duration and organic mass loss has been observed in bryozoans since the turn of the 20th century (Norman, 1903) and increased treatment duration has also been observed to cause increased loss of calcium carbonate in experiments by Tasch and Shaffer (1961), an observation also seen in the present work.

4.8 Synergy

The combined effect of bleach concentration, ultrasonication, initial wt% $MgCO_3$ in calcite and treatment duration was found to explain 78% of mass lost during treatment. Bleach concentration, ultrasonic (and associated increase in temperature and solution agitation) and the treatment duration all work together to kinetically increase the oxidation reaction of bleach with organic compounds (Moore, 2012). In addition, cavitation and shock waves caused by ultrasonication would physically abrade specimens, disrupt cells and cause damage and loss of high Mg-calcite features such as spines as can be seen in figure 8.

The combined effect of bleach concentration and treatment duration explain 17% of the wt% $MgCO_3$ in calcite lost during treatment. During the reaction of bleach with water, OH^- ions are produced, which can then capture Mg^{2+} ions from the treatment solution, pulling them out of solution and into a precipitate; this is the principal of water softening using bleach (Casiday et al., 2013). Reaction kinetics determine that the reaction will occur faster with stronger concentrations of bleach and more Mg^{2+} will be chelated during longer treatments (Moore, 2012).

5. Summary and Conclusions

Invertebrate shells and skeletons are routinely bleached using varying concentrations and durations of sodium hypochlorite for the purpose of removing organic materials in order to allow for high quality imaging, skeletal taxonomic identification and to reduce background noise in XRD analysis. The collateral damage of bleaching is that specimens may lose both Ca^{2+} and Mg^{2+} from their skeletons, and may even dissolve entirely if high concentrations of bleach or long treatment durations are used.

Ultrasonication is used for cleaning of invertebrate shells and skeletons as it physically scours the specimen with bubbles, agitates the specimen and removes loose contaminants; it is often used at the same time as bleaching. The knock-on impact of ultrasonication over longer durations is that it increases the temperature of the cleaning solution and this, in conjunction with the abrasive effects of cavitation and shock waves, can result in an increased rate of Mg^{2+} leaching, increased dissolution of inorganic carbon and loss of fragile skeletal structures. In some species where these structures are especially enhanced in magnesium, their removal could result in inaccurate skeletal characterisation.

We conclude that measurements of wt% $MgCO_3$ in skeletal calcite using XRD are not improved and do not require additional cleaning steps beyond rinsing in tap water. Historic and museum specimens often have been diligently cleaned – confidence in the accuracy of geochemical results from these specimens would be increased by knowledge of how they were treated. In the all too common absence of this historic data, care must be taken in interpreting results, particularly small-scale results that may not be greater than the error associated with bleaching, rinsing and drying, and ultrasonication.

Acknowledgements

The authors would like to thank the NHM for their support with both access to collections and the IAC unit. The authors thank the Marine Alliance for Science and Technology in Scotland (MASTS) and the Marine Environmental Research Group (MERG) at Heriot-Watt University for supporting and funding J.L.'s PhD. AMS would like to thank the University of Otago for the travel support which allowed this manuscript to be completed. JL would like to acknowledge support from the European FP7 grant [n° 315925] and UK research council knowledge exchange fellowship [NE/M006999/1] which allowed her to complete this manuscript. PK would like to thank funds from the Polish-Norwegian Research Programme operated by the National Centre for Research and Development under the Norwegian Financial Mechanism 2009-2014 in the frame of Project Contract No Pol-Nor/196260/81/2013 which allowed him to complete this manuscript. The authors would also like to thank B Metcalfe and a second anonymous reviewer for their comments which have resulted in a much improved manuscript.

References

Adams, S.J., 1968. A new cleaning technique for the preparation of calcareous fossils. Geological Magazine 105, 400-401.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P.,

2010. Chapter 4: Protein structure and function, in: Essential Cell Biology. Garland Science, New York.

Andersson, A.J., Mackenzie, F.T., Bates, N.R., 2008. Life on the margin: Implications of ocean acidification on Mg-calcite, high latitude and cold-water marine calcifiers. Mar. Ecol. Prog. Ser. 373, 265–273. doi:10.3354/meps07639

Anonymous, 1831. Manual of the practical naturalist: or, directions for collecting, preparing, and preserving subjects of Natural History. Lilly and Wait, Boston.

Arvidson, R.S., Ertan, I.E., Amonette, J.E., Luttge, A., 2003. Variation in calcite dissolution rates: Geochim. Cosmochim. Acta 67, 1623–1634. doi:10.1016/S0016-7037(02)01177-8

Bader, B., Schafer, P., 2005. Impact of environmental seasonality on stable isotope composition of skeletons of the temperate bryozoan *Cellaria sinuosa*. Palaeogeog. Palaeoclimatol. Palaeoecol. 226, 58–71.

Banta, W.C., Soule, J.D., Soule, D.F., 1973. Elements of Natural History, adapted to the present state of science, containing generic characters of nearly the while animal kingdom and descriptions of the principal species. Volume I. Chesap. Sci. 14, 62–64.

Barden, J.A., Remedios, C.G., 1985. Conformational changes in actin resulting from Ca^{2+}/Mg^{2+} exchange as detected by proton NMR spectroscopy. Eur. J. Biochem. 146, 5–8.

Barker, S., Greaves, M., Elderfield, H., 2003. A study of cleaning procedures used for foraminiferal Mg/Ca paleothermometry. Geochemistry, Geophys. Geosystems 4, 10–20. doi:10.1029/2003GC000559

Blanton, T.N., Barnes, C.L., Lelental, M., 2000. Preparation of silver behenate coatings to provide low to mid-angle diffraction calibration. J. Appl. Crystallogr. 33, 172–173.

Blanton, T.N., Huang, T.C., Toraya, H., Hubbard, C.R., Robie, S.B., Louer, D., Gobel, H.E., Will, G., Gilles, R., Raftery, T., 1995. JCPDS - International Centre for Diffraction Data round robin study of silver behenate. A possible low-angle X-ray diffraction calibration standard. Powder Diffr. 10, 91–95.

Bone, Y., James, N.P., 1997. Bryozoan stable isotope survey from the cool-water Lacepede Shelf, southern Australia. In N.P. James and J.D.A. Clarke, eds. Cool-water carbonates.

Tulsa, Oklahoma: SEPM, p. 93–105.

Borisenko, Y., Gontar, V., 1991. Biogeochemistry of skeletons of coldwater Bryozoa. Biol. Morya 1, 80–90. (in Russian).

Casiday, R., Noelken, G., Frey, R., 2013. Treating the Public Water Supply: What Is In Your Water, and How Is It Made Safe to Drink? [WWW Document]. Dep. Chem. Washingt. Univ. St Louis. URL http://www.chemistry.wustl.edu/~edudev/LabTutorials/Water/PublicWaterSupply/PublicWaterSupply.html (accessed 10.21.13).

Chedd, G., 1970. Sound: Its uses and abuses in today's technology. Aldous Books London, London.

Cheetham, A.H., Rucker, J.B. & Carver, R.E., 1969. Wall structure and mineralogy of the cheilostome bryozoan *Metrarabdotos*. Journal of Paleontology. 43, 129–135.

Chilanger, G. V, 1962. Dependence on temperature of Ca/Mg ratio of skeletal structures in organisms and direct chemical precipitates of sea water. Bull South. Calif. Acad Sci 61, 45–61.

Choquette, P.., Trusell, F.., 1978. A procedure for making the titan-yellow stain for Mg-calcite permanent. J. Sediment. Res. 48, 639–641.

Clark, D.F., 1973. Effects of ultrasound on calcareous nanofossils. Geology 1, 61–62.

Cohen, A.L., Branch, G.M., 1992. Environmentally controlled variation in the structure and mineralogy of *Patella granularis* shells from the coast of southern Africa: implications for palaeotemperature assessments. Palaeogeogr. Palaeoclimatol. Palaeoecol. 91, 49–57.

Dowsett, H.J., Haywood, A.M., Valdes, P.J., Robinson, M.M., Lunt, D.J., Hill, D.J., Stoll, D.K., Foley, K.M., 2011. Sea surface temperatures of the mid-Piacenzian Warm Period: A comparison of PRISM3 and HadCM3. Palaeogeogr. Palaeoclimatol. Palaeoecol. 309, 83–91. doi:10.1016/j.palaeo.2011.03.016

Dyrynda, P.E.J., Ryland, J.S., 1982. Reproductive strategies and life histories in the cheilostome marine bryozoans *Chartella papyracea* and *Bugula flabellata*. Mar. Biol. 256, 241–256.

Fabry, V.J., Mcclintock, J.B., Mathis, J.T., Grebmeier, J.M., 2009. Ocean acidification at high latitudes: The bellwether. Oceanography 22, 160–171.

Feldmeijer, W., Metcalfe, B., Scussolini, P., Arthur, K., 2013. The effect of chemical pretreatment of sediment on foraminifera-based proxies. Geochemistry Geophys. Geosystems 14.

Gaffey, S., Bronnimann, C., 1993. Effects of bleaching on organic and mineral phases in biogenic carbonates. J. Sediment. Petrol. 63, 752–754.

Graves, G., 1817. Naturalist's Pocket-Book, or tourist's companion, being a brief introduction to the different branches of natural history, with approved methods for collecting and preserving the various productions of nature. W and S Graves, London. doi:http://dx.doi.org/10.5962/bhl.title.11202

Groemping, U., 2013. Package " relaimpo ."

E. Raymond Hall, E,R., Russell, W.C., 1933. Dermestid Beetles as an Aid in Cleaning Bones. Journal of Mammalogy 14:4, 372–374. doi:10.1093/jmammal/14.4.372

Hall, S.R., Taylor, P.D., Davis, S. a, Mann, S., 2002. Electron diffraction studies of the calcareous skeletons of bryozoans. J. Inorg. Biochem. 88, 410–9.

Hayward, P., Ryland, J., 1998. Cheilostomatous Bryozoa Part I: Aeteoidea - Cribrilinoidea, second. ed. Fields Study Council, Shrewsbury.

Hefti, E., Trechsel, U., Rüfenacht, H., Fleisch, H., 1980. Use of dermestid beetles for cleaning bones. Calcified Tissue International

Hodgkinson, R.L., 1991. Microfossil processing: a damage report. Micropaleontology 37, 320–326.

Inoue, H., Yuasa-Hashimoto, N., Suzuki, M., Nagasawa, H., 2008. Structural determination and functional analysis of a soluble matrix protein associated with calcification of the exoskeleton of the crayfish, *Procambarus clarkii*. Biosci. Biotechnol. Biochem. 72, 2697–2707. doi:10.1271/bbb.80349

Intergovernmental Oceanographic Commission (IOC) of UNESCO, 2010. The Ocean Biogeographic Information System [WWW Document]. URL www.iobis.org (accessed

1.7.13).

Kamba A.S., Ismail. M., Ibrahim. T.A.T., Zakaria. Z.A.B., 2013. Synthesis and Characterisation of Calcium Carbonate Aragonite Nanocrystals from Cockle Shell Powder (*Anadara granosa*). Journal of Nanomaterials 2013, 9. http://dx.doi.org/10.1155/2013/398357

Keatings, K.W., Holmes, J.A., Heaton, T.H.E., 2006. Effects of pre-treatment on ostracod valve chemistry. Chem. Geol. 235, 250–261. doi:10.1016/j.chemgeo.2006.07.003

Klein, D., 2012. Organic chemistry. John Wiley & Sons, Ltd.

Krause-Nehring, J., Klugel, A., Nehrke, G., Brellochs, B., Brey, T., 2011. Impact of sample pre-treatment on the measured element concentrations in the bivalve *Arctica islandica*. Geochemistry Geophys. Geosystems 12.

Kuklinski, P., Taylor, P.D., 2009. Mineralogy of Arctic bryozoan skeletons in a global context. Facies 55, 489–500. doi:10.1007/s10347-009-0179-3

Lindeman, R.H., Merenda, P.F., Gold, R.Z., 1980. Introduction to Bivariate and Multivariate Analysis. Scott Foresman, Glenview, IL.

Lombardi, C., Rodolfo-Metalpa, R., Cocito, S., Gambi, M.C., Taylor, P.D., 2011. Structural and geochemical alterations in the Mg calcite bryozoan *Myriapora truncata* under elevated seawater pCO2 simulating ocean acidification. Mar. Ecol. 32, 1–11. doi:10.1111/j.1439-0485.2010.00426.x

Lowenstam, H.A., 1954. Environmental relations of modification compositions of certain carbonate secreting marine invertebrates. Proc. Natl. Acad. Sci. U. S. A. 40, 39–48.

Loxton, J., 2014. Investigations into the Skeletal Mineralogy of Temperate and Polar Bryozoans. Heriot Watt University. doi:10.13140/RG.2.2.36799.92326

Loxton, J., Kuklinski, P., Mair, J.M., Spencer Jones, M., Porter, J.S., 2012. Patterns of magnesium-calcite distribution in the skeleton of some polar bryozoan species. In A. Ernst, P. Schäfer, and J. Scholz, eds. Bryozoan Studies 2010. Berlin, Heidelberg: Springer Berlin Heidelberg, p. 169–185.

Loxton, J., Kuklinski, P., Barnes, D.K.A., Najorka, J., Spencer Jones, M., Porter, J.S., 2014a. Variability of Mg-calcite in Antarctic bryozoan skeletons across spatial scales. Mar. Ecol. Prog. Ser. 507, 169-180.

Loxton, J., Kuklinski, P., Najorka, J., Spencer Jones, M., Porter, J.S., 2014b. Variability in the skeletal mineralogy of temperate bryozoans: the relative influence of environmental and biological factors. Mar Ecol. Prog. Ser. 510, 45-57.

Machiyama, H., Yamada, T., Kaneko, N., Iryu, Y., Odawara, K., Asami, R., Matsuda, H., Mawatari, S.F., Bone, Y., James, N.P., 2003. Carbon and Oxygen isotopes of cool-water bryozoans from the great Australian bight and their paleoenvironmental significance. Proceedings of the Ocean Drilling Program, Scientific Results 182.

Mackenzie, F.T., Bischoff, W.D., Bishop, F.C., Loijens, M., Schoon-Maker, J., Wollast, R., 1983. Magnesian calcites: low temperature occurence, solubility and solid-solution behaviour., in: Reeder, R.J. (Ed.), Carbonates: Mineralogy and Chemistry. Vol 11. Mineralogical Society of America, pp. 97–143.

Marr, J.P., Bostock, H.C., Carter, L., Bolton, A., Smith, E., 2013. Differential effects of cleaning procedures on the trace element chemistry of planktonic foraminifera. Chem. Geol. 351, 310–323. doi:10.1016/j.chemgeo.2013.05.019

Mitsuguchi, T., Uchida, T., Matsumoto, E., Isdale, P., Kawana, T., 2001. Variations in Mg/Ca, Na/Ca & Sr/Cr ratios of coral skeletons with chemical treatments: implications for chemical geochemistry. Geochim. Cosmochim. Acta 25, 2865–2874.

Moore, J.T., 2012. The lowdown on kinetics: tortoise or the hare?, in: Chemistry II for Dummies. John Wiley & Sons, Ltd., pp. 99–123.

Norman, A.M., 1903. Notes on the Natural History of East Finmark. Polyzoa (continued). Ann. Mag. Nat. Hist 7, 87–128.

Oomori, T., Kaneshima, H., Maezato, Y., Kitano, Y., 1987. Distribution coefficient of Mg^{2+} ions between calcite and solution and 10-50ºC. Mar. Chem. 20, 327–336.

Poluzzi, A., Sartori, R., 1973. Carbonate Mineralogy of some bryozoa from Talbot Shoal. Giornale di Geologia 39, 11–15.

Poluzzi, A., Sartori, R., 1974. Report on the carbonate mineralogy of Bryozoa. Docum. Lab.

Geol. Fac. Sci. Lyon 3, 193–210.

Piwoni-Piórewicz, A., Kukliński, P., Strekopytov, S., Humphreys-Williams, E., Najorka, J., Iglikowska, A., 2017. Size effect on the mineralogy and chemistry of *Mytilus trossulus* shells from the southern Baltic Sea: implications for environmental monitoring. Environ. Monit. Assess. 189, 197. doi:10.1007/s10661-017-5901-y

R Core Team, 2013. R: A Language and Environment for Statistical Computing.

Ries, J.B., Cohen, A.L., Mccorkle, D.C., 2009. Marine calcifiers exhibit mixed responses to CO_2 -induced ocean acidification. Geol. Soc. Am. 37, 1131–1134. doi:10.1130/G30210A.1

Ripley, B., 2013. Package "boot."

Robertson, B.Y.J.D., 1961. Studies on the chemical composition of muscle tissue II the abdominal flexor muscles of the lobster Nephrops norvegicus. J. Exp. Biol. 38, 707–728.

Roger, K.D., Daniels P. (2002) An X-ray diffraction study of the effects of heat treatment on bone mineral microstructure. Biomaterials 23, 2577-2585.

Rucker, J.B., Carver, R.E., 1969. A survey of the carbonate mineralogy of cheilostome Bryozoa. Journal of Paleontology, 791-799.

Rutzky, I.S., Elver, W.B., Maisey, J.G., Kellner, A.W.A., 1994. Chapter 7. Chemical preparation techniques in Vertebrate Paleontological Techniques. Cambridge University Press, UK.

Sandberg, P.A., 1971. Scanning electron microscopy of cheilostome bryozoan skeletons; techniques and preliminary observations. Micropaleontology 17, 129–151.

Sandberg, P.A., 1973. Degree of Individuality in Cheilostome Bryozoa: Skeletal Criteria. In R.S. Boardman, A.H. Cheetham, O.C.N, eds. Animal Colonies. Stroudsburg, PA, USA: Dowden, Hutchinson & Ross Inc., p. 305–315.

Sandberg, P.A., 1975. Bryozoan diagenesis: Bearing on the nature of the original skeleton of rugose corals. Journal of Paleontology 49, 587–606.

Sandberg, P.A., 1977. Ultrastructure, mineralogy and development of bryozoan skeletons. In R.M. Woollacott, R.L. Zimmer, eds. Biology of Bryozoans. New York: Academic Press, p. 143–177.

Sandberg, P.A., 1983. Ultrastructure and skeletal development in cheilostomate bryozoa. In R.S. Boardman et al., eds. Treatise on Invertebrate Paleontology Part G Bryozoa, revised volume 1. Boulder, Colorado: Geological Society of America, p. 238–286.

Santagata, S., 2007. The morphology and evolutionary significance of the ciliary fields and musculature among marine bryozoan larvae. J. Morphol. 269, 349–364. doi:10.1002/jmor

Schäfer, P., Bader, B., 2008. Geochemical composition and variability in the skeleton of the bryozoan *Cellaria sinuosa* (Hassall): Biological versus environmental control. In S.J. Hageman, M.M. Key, J.E. Winston, eds. Proceedings of the 14th International Bryozoology Association conference. Martinsville, Virginia: Virginia Museum of Natural History Publications, p. 269–279.

Schopf, T.J.M., Allan, J.R., 1970. Phylum Ectoprocta, Order Cheilostomata: microprobe analysis of calcium, magnesium, strontium and phosphorus in skeletons. Science (80-.). 169, 280–282.

Simonsen, K.P., Rasmussen, A.R., Mathisen, P., Petersen, H., Borup, F., 2011. A Fast Preparation of Skeletal Materials Using Enzyme Maceration. Journal of Forensic Sciences 56:2, 480–484. **DOI: 10.1111/j.1556-4029.2010.01668.x**

Sjoberg, E.L., Rickard, D.T., 1984. Temperature dependence of calcite dissolution kinetics between 1 and 62ºC at pH 2.7 to 8.4 in aueous solutions. Geochim. Cosmochim. Acta 48, 485–493.

Smith, A.M., 2007. Age, growth and carbonate production by erect rigid bryozoans in Antarctica. Palaeogeogr. Palaeoclimatol. Palaeoecol. 256, 86–98. doi:10.1016/j.palaeo.2007.09.007

Smith, A.M., Clark, D.E., 2010. Skeletal Carbonate Mineralogy of Bryozoans From Chile: an Independent Check of Phylogenetic Patterns. Palaios 25, 229–233.

Smith, A.M., Girvan, E., 2010. Understanding a bimineralic bryozoan: Skeletal structure and carbonate mineralogy of *Odontionella cyclops* (Foveolariidae: Cheilostomata: Bryozoa)

in New Zealand. Palaeogeogr. Palaeoclimatol. Palaeoecol. 289, 113–122. doi:10.1016/j.palaeo.2010.02.022

Smith, A.M., Key, M.M., 2004. Controls, variation, and a record of climate change in detailed stable isotope record in a single bryozoan skeleton. Quat. Res. 61, 123–133. doi:10.1016/j.yqres.2003.11.001

Smith, A.M., Lawton, E.I., 2010. Growing up in the temperate zone: Age, growth, calcification and carbonate mineralogy of *Melicerita chathamensis* (Bryozoa) in southern New Zealand. Palaeogeogr. Palaeoclimatol. Palaeoecol. 298, 271–277.

Smith, A.M., Key, M.M., Henderson, Z.O.E.E., Davis, V.C., Winter, D.J., 2016. Pretreatment for removal of organic material is not necessary for X-ray-diffraction determination of mineralogy in temperate skeletal carbonate. J. Sediment. Res. 86, 1425–1433.

Smith, A.M., Nelson, C., Danaher, P., 1992. Dissolution behaviour of bryozoan sediments: taphonomic implications for nontropical shelf carbonates. Palaeogeogr. Palaeoclimatol. Palaeoecol. 93, 213–226. doi:10.1016/0031-0182(92)90098-P

Smith, A.M., Nelson, C., Spencer, H., 1998. Skeletal carbonate mineralogy of New Zealand bryozoans. Mar. Geol. 151, 27–46. doi:10.1016/S0025-3227(98)00055-3

Stanley, S., Hardie, L., 1998. Secular oscillations in the carbonate mineralogy of reef-building and sediment-producing organisms driven by tectonically forced shifts in seawater chemistry. Palaeogeogr. Palaeoclimatol. Palaeoecol. 144, 3–19. doi:10.1016/S0031-0182(98)00109-6

Steedman, H.F., 1976. Zooplankton fixation and preservation, in: Monographs on Oceanograpy Methodology 1-4. UNESCO, Paris.

Stevens, C.H., Jones, D.H., Todd, R.G., 1960. Ultrasonic vibrations as a cleaning agent for fossils. Journal of Paleontology, 727-730.

Tasch, P., Shaffer, B.L., 1961. Study of scolecodonts by transmitted light. Micropaleontology 7, 369–371.

Tavener-Smith, R., Williams, A., 1972. The secretion and structure of the skeleton of living and fossil Bryozoa. Philos. Trans. R. Soc. B Biol. Sci. 264, 97–211.

Taylor, P.D., James, N.P., Bone, Y., Kuklinski, P., Kyser, T.K., 2009. Evolving mineralogy of cheilostome bryozoans. Palaios 24, 440–452. doi:10.2110/palo.2008.p08

Taylor, P.D., Weedon, M.J., 2000. Skeletal ultrastructure and phylogeny of cyclostome bryozoans. Zool. J. Linn. Soc. 337–399. doi:10.1006/zjls.1999.0195

Toombs, H.A., Rixon, A.E., 1959. The use of acids in the preparation of vertebrate fossils. Curator: The Museum Journal.

Voltcraft, 2017. Voltcraft pH 100ATC pH Messq: datasheet Version 0.1. [WWW Document]. URL www.conrad-electronic.co.uk/ce/en/product/101145/Voltcraft-PH-100-ATC-pH-Meter (accessed 14.9.17).

Walker, A.K., Fitton, M.G., Vane-Wright, R.I., Carter, D.J., 1999. Insects and other invertebrates, in: Carter, D., Walker, A.K. (Eds.), Care and Conservation of Natural History Collections. Butterworth Heinemann, Oxford, pp. 37–60.

Watanabe, T., Minagawa, M., Oba, T., Winter, A., 2001. Pre-treatment of coral aragonite for Mg and Sr analysis: implications for coral thermometers. Geochem J. 35, 265–269.

Zuschin, M., Stachowitsch, M., Stanton, R.J., 2003. Patterns and processes of shell fragmentation in modern and ancient marine environments. Earth-Science Rev. 63, 33–82. doi:10.1016/S0012-8252(03)00014-X

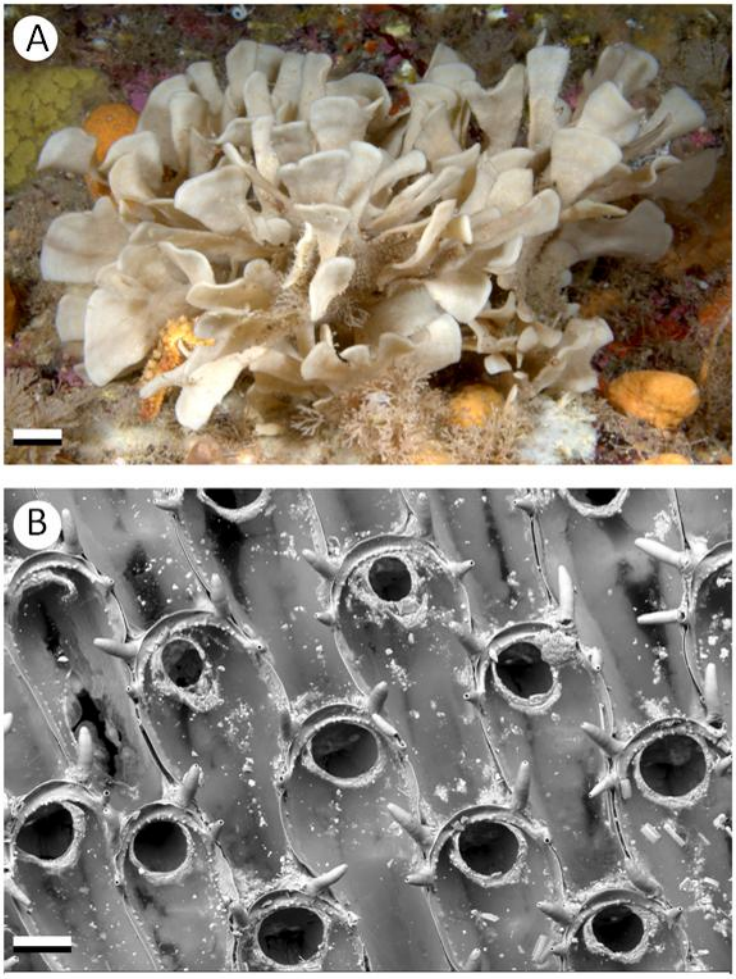


Fig. 1

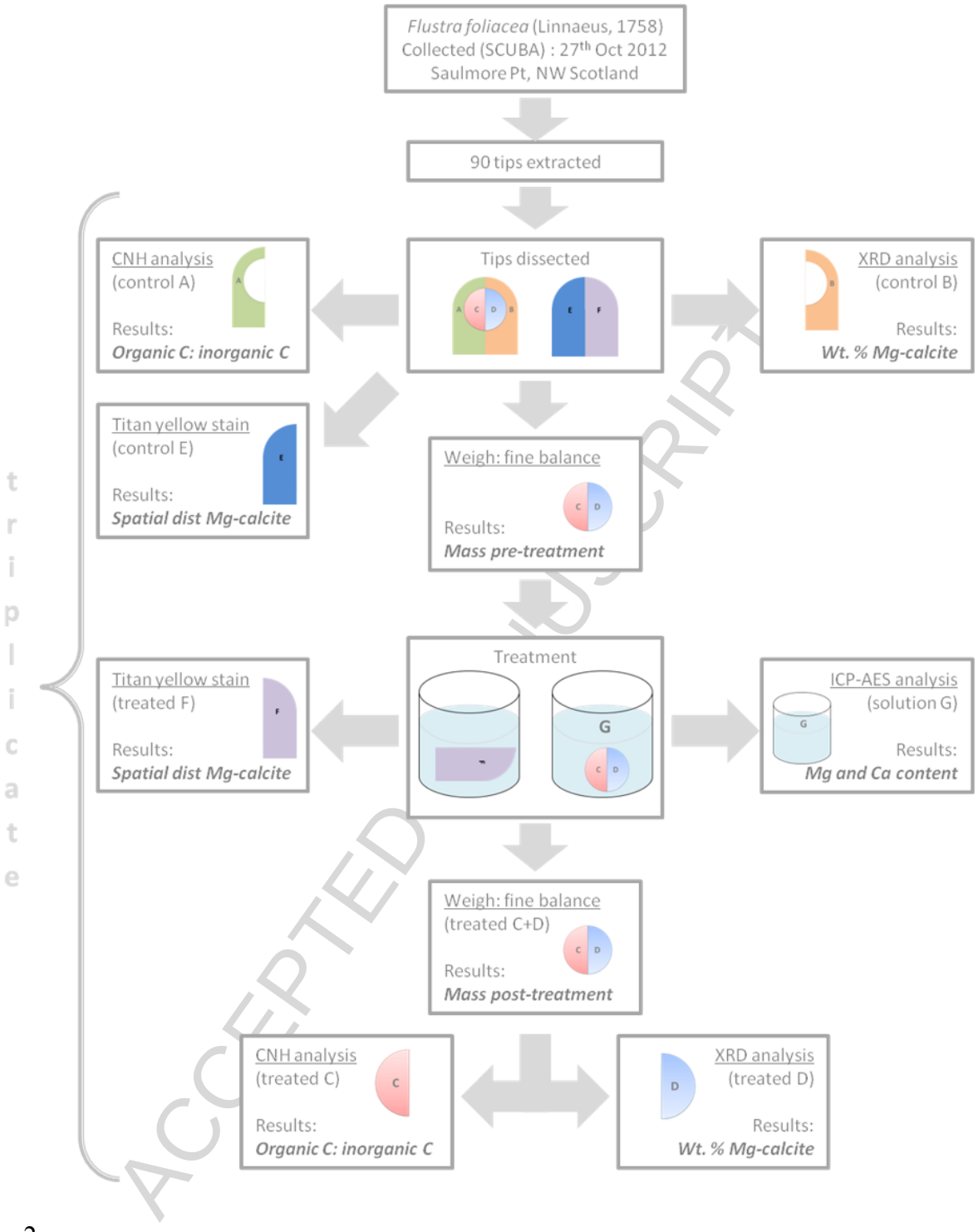


Fig. 2

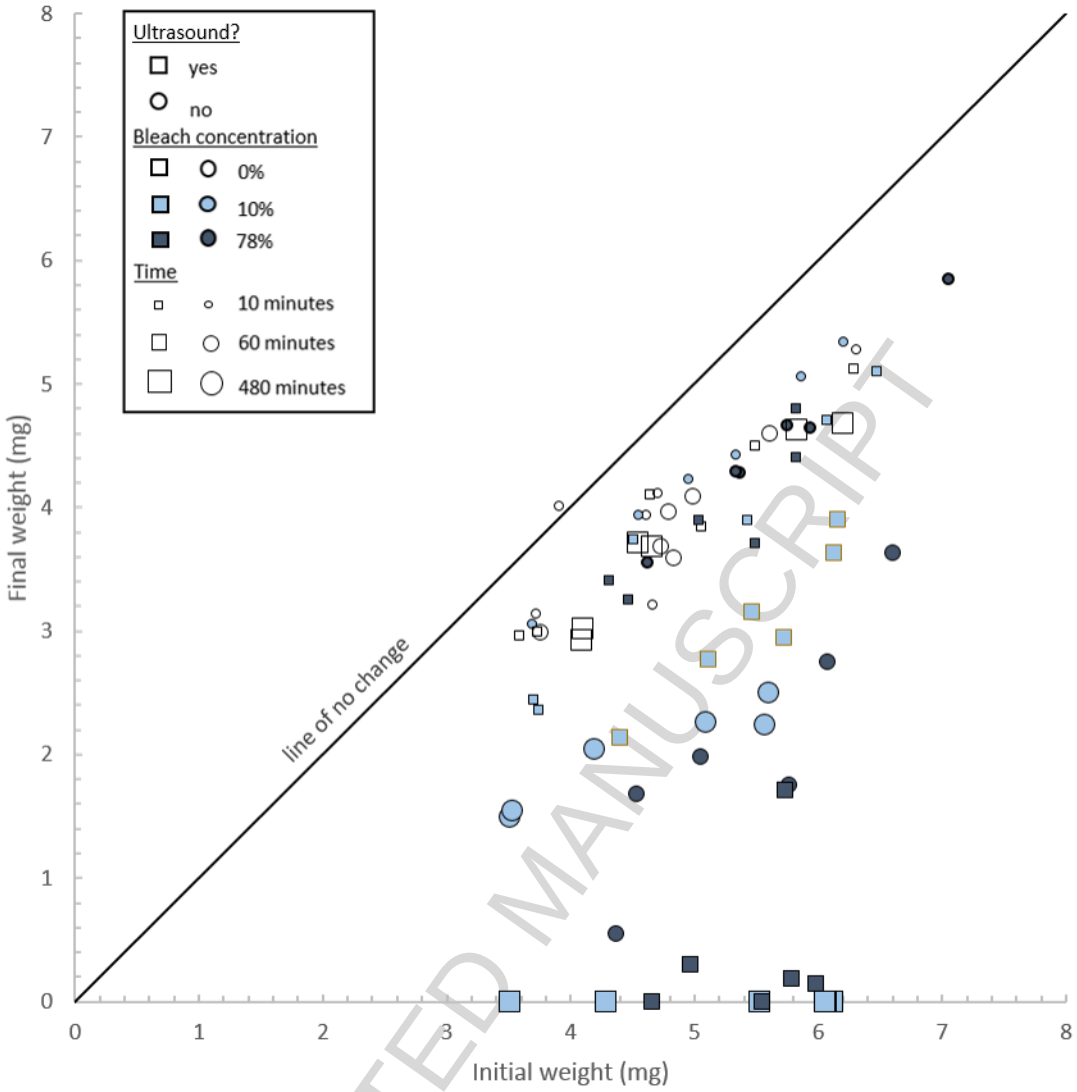


Fig. 3

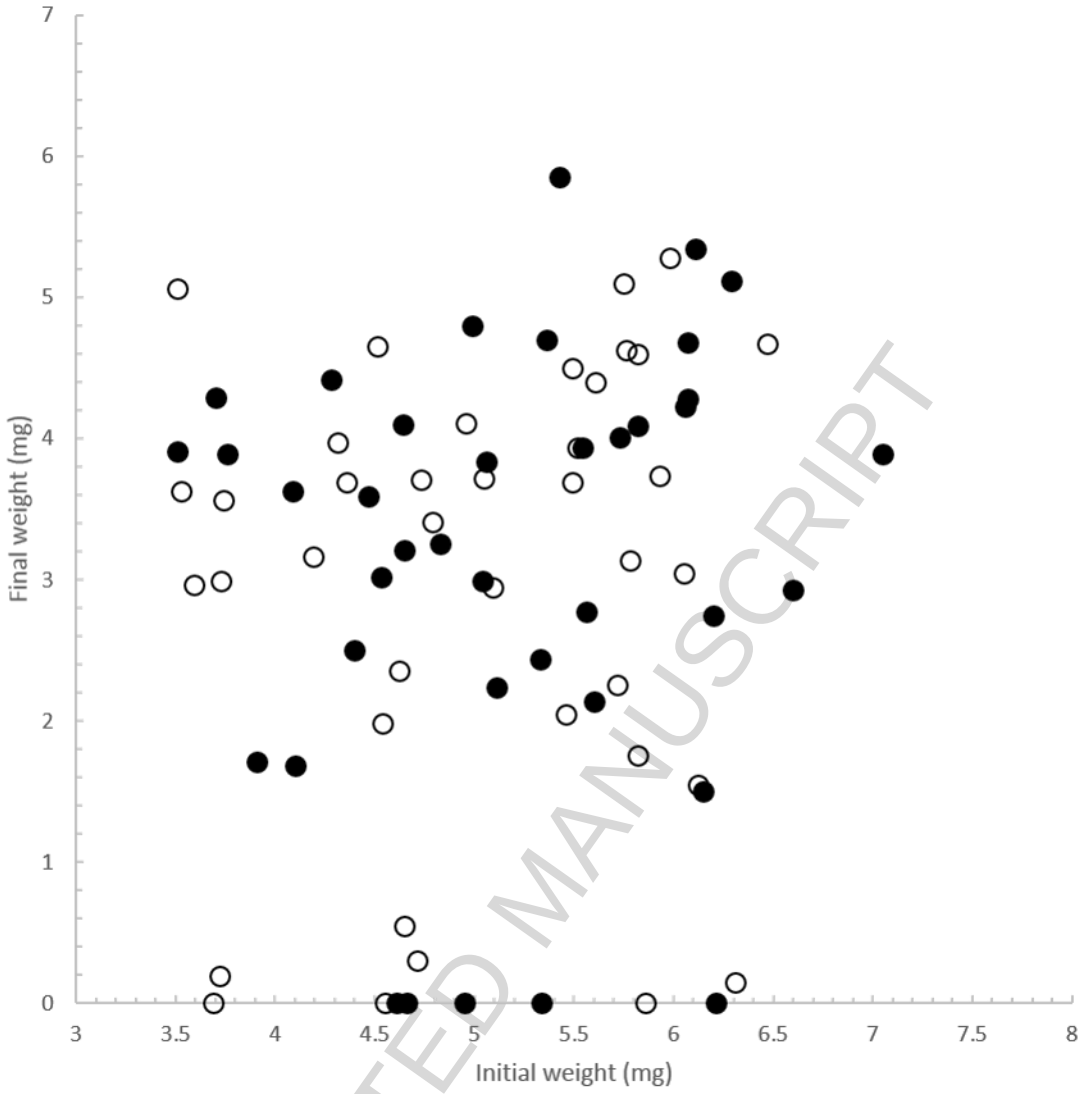


Fig. 4

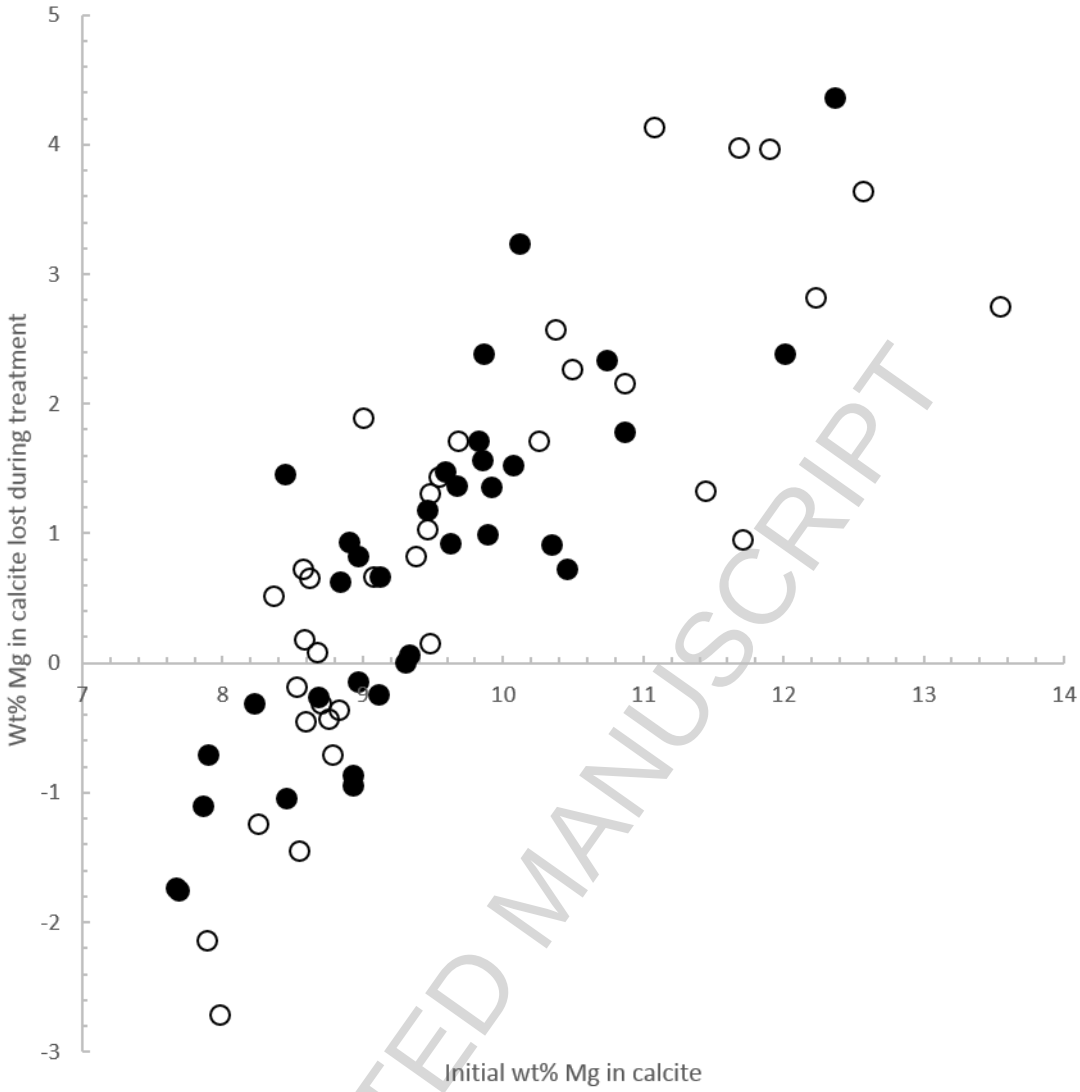
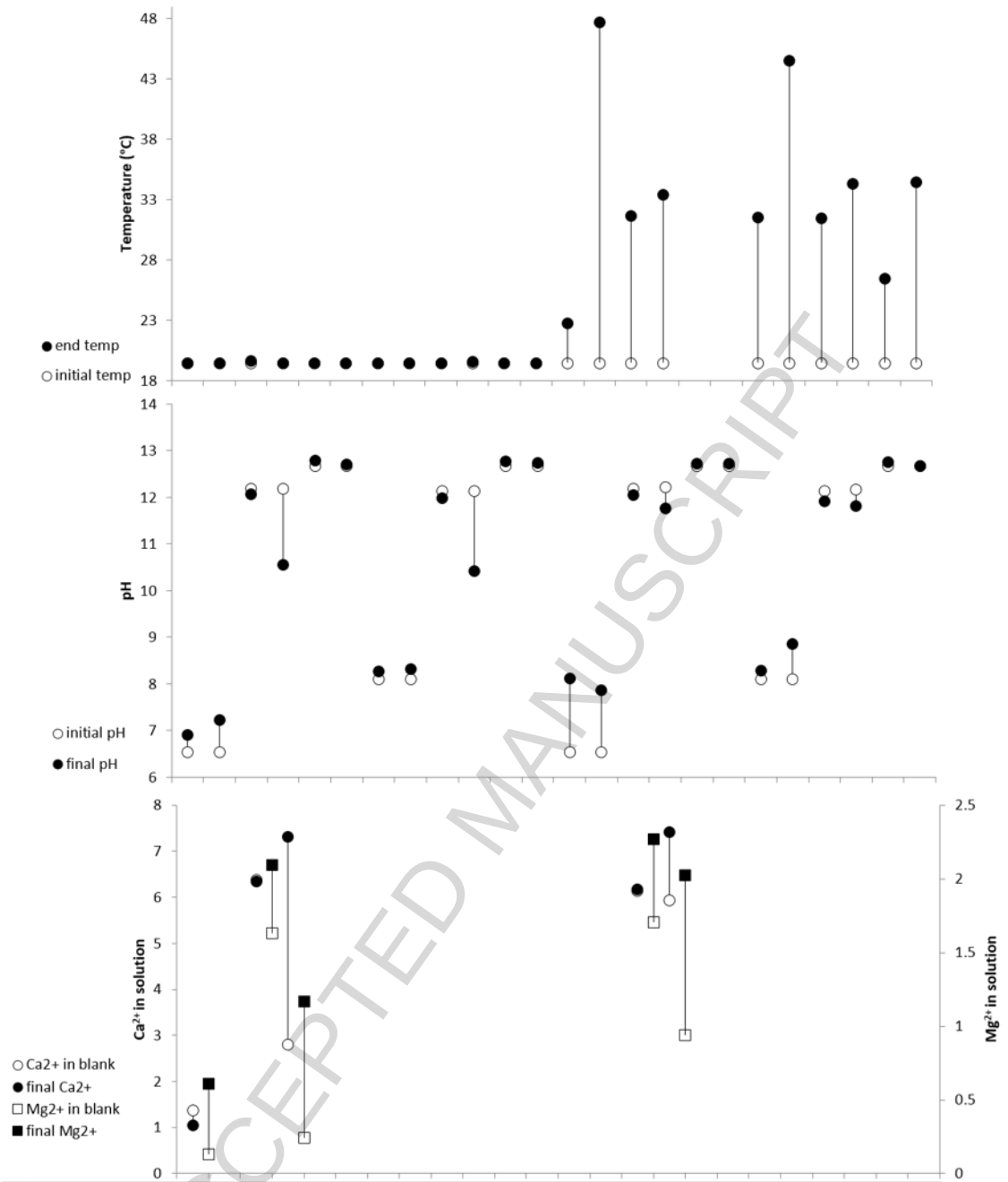


Fig. 5



Treatment																							
Ultrasound:	NO ULTRASOUND												ULTRASOUND										
Water source:	DEIONIZED						TAP						DEIONIZED				TAP						
Bleach conc'n (%):	0%		10%		78%		0%		10%		78%		0%		10%		78%		0%		10%		78%	
Time (minutes):	10	480	10	480	10	60	10	480	10	480	10	60	10	480	10	60	10	60	10	480	10	60	10	60

Fig. 6

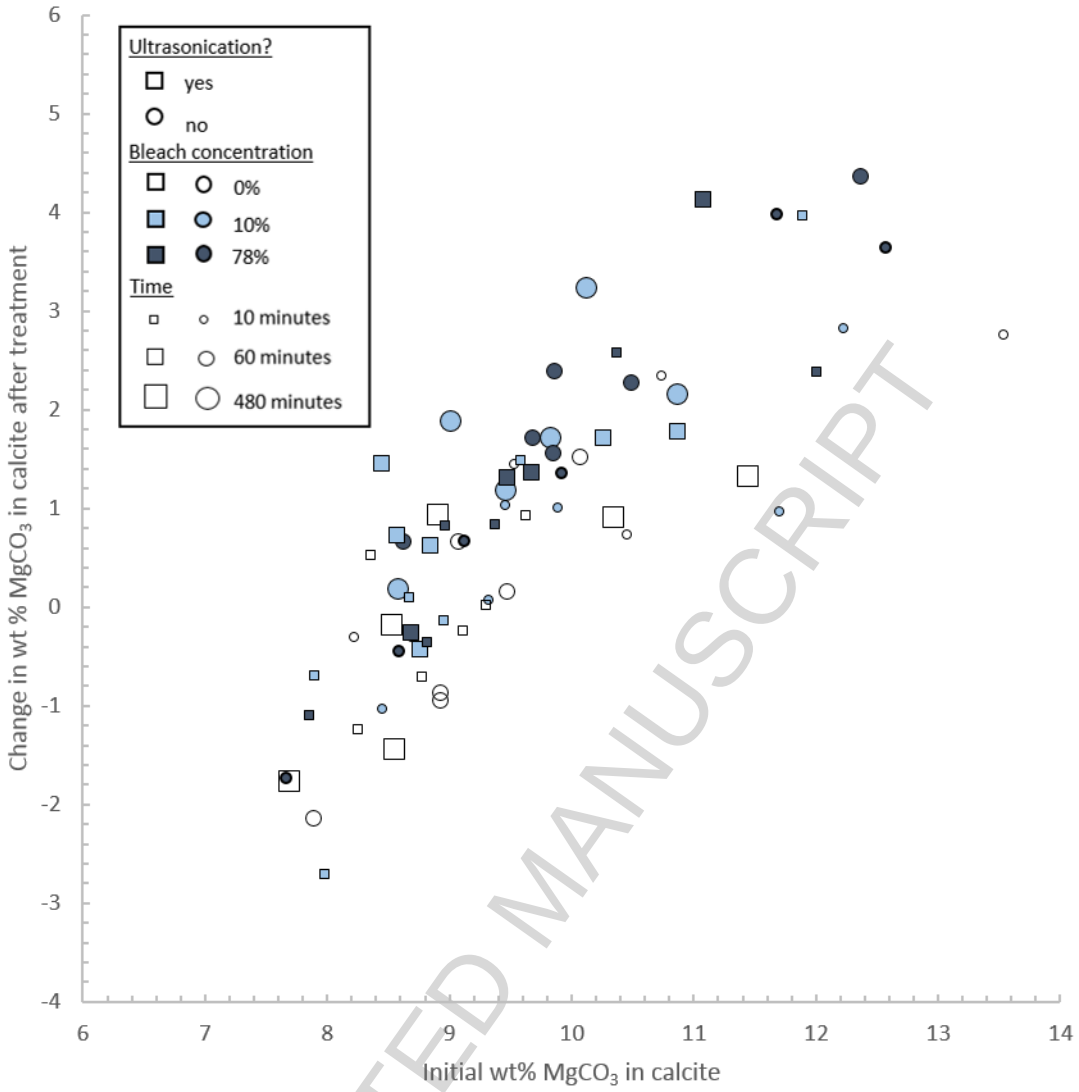


Fig. 7

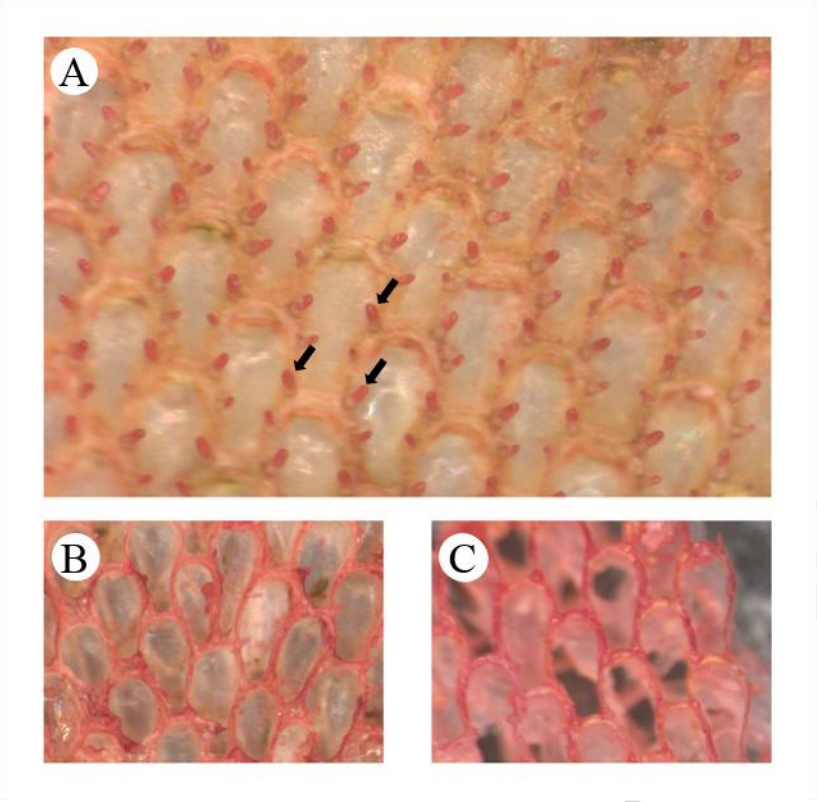


Fig. 8